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Detection of Cariogenic *Streptococcus mutans* in Extirpated Heart Valve and Atheromatous Plaque Specimens

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The involvement of oral bacteria in the pathogenesis of cardiovascular diseases has been the focus of attention in many studies, and several periodontal pathogens have been detected in diseased cardiovascular lesions, suggesting relationships between oral microorganisms and cardiovascular diseases. However, no information is available regarding the involvement of cariogenic pathogens such as *Streptococcus mutans*. The presence of oral streptococcal species and periodontitis-related bacteria in 35 heart valve and 27 atheromatous plaque clinical specimens, as well as 32 dental plaque specimens from the same subjects, was analyzed using a PCR method. Furthermore, broad-range PCR with DNA sequencing analysis was employed to identify the bacterial species in those samples. *Streptococcus mutans* was frequently detected in the heart valve (69%) and atheromatous plaque (74%) specimens, while other bacterial species, including those related to periodontitis, were detected with much lower frequencies. The bacterial composition in cardiovascular tissues was found to be markedly distinct from that in dental plaque, with only a limited number of species, including *S. mutans*, in the cardiovascular regions shown to have possibly originated from the oral cavity. Semiquantitative assay results revealed that *S. mutans* was detected in significant quantities in the heart valve (40%) and atheromatous plaque (48%) specimens, whereas the quantities of all other tested bacterial species, including several related to periodontitis, were negligible in the cardiovascular samples. These results indicate that *S. mutans* is a possible causative agent of cardiovascular disease.

Accumulated evidence suggests that oral bacterial pathogens are associated with several kinds of systemic diseases, such as infective endocarditis (IE), cardiovascular diseases, bacterial pneumonia, low birth weight, and diabetes mellitus (12). Those associations are speculated to be initiated by transient or prolonged bacteremia caused by oral infection: i.e., from professional dental treatments and daily oral care practices such as tooth brushing and flossing, as well as from food chewing, which possibly induces dissemination of oral bacteria into the bloodstream (21). Oral streptococcal species are major components of the oral microflora that are known to occasionally cause bacteremia and IE (13). *Streptococcus mutans*, a major pathogenic agent of dental caries, has also been isolated from the blood of patients with IE, strongly suggesting a close relationship of the pathogen with IE (5, 23, 24).

The recent development of several molecular techniques has enabled prompt identification of targeted bacterial species in specimens with significantly improved specificity and sensitivity. PCR methods using primers constructed with a species-specific nucleotide alignment are widely used for the detection of specific species. In addition, broad-range eubacterial PCR with amplification of bacterial DNA and subsequent direct sequencing is considered to be a reliable diagnostic tool (18, 19).

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Dental caries and chronic marginal periodontitis are two major infectious diseases clinically encountered in the field of dentistry. Recently, several studies have reported detection of periodontal pathogens in cardiovascular specimens from patients using a PCR method, suggesting relationships between oral microorganisms and systemic cardiovascular diseases (20). However, there are no reports of oral streptococci detected in those tissues, especially regarding cariogenic *S. mutans*, which is a potential cause of IE. In the present study, we analyzed the presence of streptococcal species in diseased heart valve and atheromatous plaque specimens, as well as in dental plaque samples from the same subjects.

MATERIALS AND METHODS

Specimens. A total of 35 heart valve specimens from 24 males and 11 females (average age, 67.4 years old; range, 46 to 84 years) and 27 atheromatous plaque specimens from 22 males and 5 females (average age, 70.6 years old; range, 45 to 81 years) were collected according to a protocol approved by the Ethics Committee of Osaka Rosai Hospital. All of the heart valve tissue specimens were excised during a valve replacement procedure, following diagnosis of aortic regurgitation, mitral regurgitation, or tricuspid regurgitation, while the atheromatous plaque specimens were collected during treatment for a thoracic or abdominal aortic aneurysm. These specimens were aseptically obtained at the Department of Cardiovascular Surgery, Osaka Rosai Hospital, Sakai, Osaka, Japan, from December 2004 to November 2005. In addition, supra- and subgingival plaque samples from 32 of those patients were taken from the mesial and buccal subgingival sites of all teeth with sterile Gracey curettes at the Department of Dentistry and Oral Surgery of Osaka Rosai Hospital prior to the cardiovascular operations.

PCR detection of oral streptococci and periodontitis-related bacteria. Whole DNA fractions were extracted from the heart valve and atheromatous plaque

TABLE 1. PCR primers used for identification of oral streptococci and periodontitis-related bacteria

Bacterial species	Primer pair sequences (5′ to 3′)	Annealing temp (°C)	Expected size (bp)	Reference
Oral streptococci				
S. mutans	GGCACCACAACATTGGGAAGCTCAGTT GGAATGGCCGCTAAGTCAACAGGAT	70	433	9
S. sobrinus	GATGATTTGGCTCAGGATCAATCCTC ACTGAGCCAGTAGTAGACTTGGCAACT	70	328	9
S. salivarius	GTGTTGCCACATCTTCACTCGCTTCGG CGTTGATGTGCTTGAAAGGGCACCATT	66	544	9
S. sanguinis	GGATAGTGGCTCAGGGCAGCCAGTT GAACAGTTGCTGGACTTGCTTGTC	70	313	9
S. oralis	TCCCGGTCAGCAAACTCCAGCC GCAACCTTTGGATTTGCAAC	66	374	9
S. gordonii	CTATGCGGATGATGCTAATCAAGTG GGAGTCGCTATAATCTTGTCAGAAA	70	440	9
Periodontitis-related bacteria				
P. gingivalis	TGTAGATGACTGATGGTGAAAACC ACGTCATCCCCACCTTCCTC	60	197	22
P. intermedia	TTTGTTGGGGAGTAAAGCGGG TCAACATCTCTGTATCCTGCGT	55	575	2
T. denticola	AAGGCGGTAGAGCCGCCGCTCA AGCCGCTGTCGAAAAGCCCA	55	311	25
T. forsythia	GCGTATGTAACCTGCCCGCA TCGTTCAGTGTCAGTTATACCT	60	641	2
A. actinomycetemcomitans	CTAGGTATTGCGAAACAATTTG CCTGAAATTAAGCTGGTAATC	55	262	6
C. rectus	TTTCGGAGCGTAAACTCCTTTTC TTTCTGCAAGCAGACACTCTT	60	598	2

specimens, after being aseptically cut into small pieces, as well as from the dental plaque samples using a method described previously (16). Thereafter, a PCR method using specific primer sets for the glucosyltransferase gene was employed to detect six oral streptococcal species (*S. mutans*, *Streptococcus sobrinus*, *Streptococcus salivarius*, *Streptococcus sanguinis*, *Streptococcus oralis*, and *Streptococcus gordonii*) as described previously (9). Briefly, PCR was carried out in 20 μl of a reaction mixture containing 0.5 U of TaKaRa Ex *Taq* (TAKARA BIO, Inc., Otsu, Shiga, Japan), 0.5 μM of oligonucleotide primers, template DNA, and 1.5 mM of $MgCl_2$, according to the manufacturer's protocols. Amplification was performed with a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, Calif.) using the following parameters: 30 cycles of a denaturing step at 98°C for 10 s and primer-annealing and extension steps at 66°C or 70°C for 1 min. The primer-annealing and extension temperatures were changed for each primer set (Table 1). *S. mutans* MT8148, *S. sobrinus* 6715, *S. salivarius* HHT, *S. sanguinis* ATCC 10556, *S. oralis* ATCC 10557, and *S. gordonii* ATCC 10558 were used as positive controls. In addition, the periodontitis-related bacterial species *Porphyromonas gingivalis*, *Prevotella intermedia*, *Treponema denticola*, *Tannerella forsythia*, *Actinobacillus actinomycetemcomitans*, and *Campylobacter rectus* were analyzed using a PCR method, as described previously (1). This PCR assay was done by the method described above, except for the thermal cycles, which were as follows. Initial denaturation was performed at 95°C for 30 s, followed by 55°C or 60°C for 30 s and 72°C for 1 min, with a final extension step at 72°C for 7 min. The annealing temperature was varied depending on the primer set used (Table 1).

Identification of bacterial species. A broad-range PCR technique targeting 16S rRNA with direct sequencing was carried out to quantitatively identify bacterial species in the specimens. 16S rRNA was amplified by PCR using AmpliTaq Gold polymerase (Applied Biosystems) with the broad-range 16S rRNA primers 536f and 1050r (19). Next, the PCR products were separated by electrophoresis on 1.5% agar gel and the amplified DNA fragments were extracted using a QIAEX II gel extraction kit (QIAGEN Sciences, Düsseldorf, Germany). The extracted DNA was directly cloned into a pGEM-T Easy vector (Promega, Madison, Wis.), and then 10 clones from each sample were randomly chosen and analyzed by dye terminator reaction with a DNA sequencing system (ABI PRISM 310 Genetic Analyzer; Applied Biosystems) and the BigDye terminator cycle sequencing kit. Data analysis was performed using Gene Works software (IntelliGenetics, Mountain View, Calif.). To identify the bacterial species, the 16S rRNA sequences were compared with those available in the GenBank, EMBL, and DDBJ databases, using the gapped BLASTN 2.0.5 program obtained from the National Center for Biotechnology Information server (http:/www.ncbi.nlm.nih.gov/BLAST/). Identification at the species level was determined when the 16S rRNA sequence similarity was greater than 99% of that of the GenBank prototype strain sequence, while identification at the genus level was defined as a 16S rRNA sequence similarity of 97% with that of the GenBank prototype strain sequence. The number of species identified in the 10 random clones was converted to a semiquantitative amount of species per sample.

Statistical analysis. Fisher's exact probability test was used for statistical analyses of the comparative frequencies of bacterial occurrence. A P value of <0.05 was considered significant.

RESULTS

Distribution of streptococcal species and periodontitis-related bacteria. The bacterial compositions in the 62 cardiovascular specimens (35 heart valve and 27 atheromatous plaque

TABLE 2. Detection of oral streptococci and periodontitis-related bacteria in heart valve, atheromatous plaque, and dental plaque samples by PCR

Bacterial species	No. (%) of positive samples[a]			
	Heart valve tissue ($n = 35$)	Dental plaque ($n = 18$)	Atheromatous plaque ($n = 27$)	Dental plaque ($n = 14$)
Oral streptococci				
S. mutans	24 (68.6)	16 (88.9)	20 (74.1)	14 (100)
S. sobrinus	0 (0)*	0 (0)*	1 (3.7)*	1 (7.1)*
S. salivarius	0 (0)*	3 (16.7)*	1 (3.7)*	3 (21.4)*
S. sanguinis	6 (17.1)*	14 (77.8)	7 (25.9)*	12 (85.7)
S. oralis	0 (0)*	13 (72.2)	1 (3.7)*	10 (71.4)
S. gordonii	0 (0)*	1 (5.6)*	0 (0)*	1 (7.1)*
Periodontitis-related bacteria				
P. gingivalis	4 (11.4)*	10 (55.6)*	2 (7.4)*	8 (57.1)*
P. intermedia	2 (5.7)*	3 (16.7)*	1 (3.7)*	3 (21.4)*
T. denticola	14 (40.0)*	11 (61.1)	12 (44.4)	12 (85.7)
T. forsythia	0 (0)*	17 (94.4)	0 (0)*	12 (85.7)
A. actinomycetemcomitans	9 (25.7)*	8 (44.4)*	7 (25.9)*	7 (50.0)*
C. rectus	2 (5.7)*	15 (83.3)	1 (3.7)*	14 (100)

[a] *, significant difference ($P < 0.05$) compared to *S. mutans*.

specimens) were compared to those in dental plaque samples from the same subjects (Table 2). *S. mutans* was most prevalent in the heart valve tissue (68.6%) and atheromatous plaque (74.1%) specimens and was also significantly distributed in the dental plaque samples (88.9 to 100%). In contrast, *S. sanguinis* had the second greatest occurrence in dental plaque (77.8 to 85.7%), while it was detected at a low frequency in the heart valve (17.1%) and atheromatous plaque (25.9%) specimens. The other species, including *S. sobrinus*, *S. salivarius*, and *S. oralis*, were rarely detected in the heart valve and atheromatous plaque specimens, though *S. oralis* and *S. salivarius* were prevalent in dental plaque at ratios ranging from 71.4% to 72.2% and from 16.7% to 21.4%, respectively. As for periodontitis-related bacteria, *T. denticola* was the most frequently detected species in heart valve tissues (40.0%) and atheromatous plaque (44.4%). *A. actinomycetemcomitans*, the second most frequently detected species, was detected in 25.7% of the heart valves and 25.9% of atheromatous plaque samples. In addition, the detection frequencies of *P. gingivalis*, *P. intermedia*, and *C. rectus* were extremely low in both the heart valve and atheromatous plaque specimens, while *T. forsythia* was not detected in any of those, in spite of its high detection frequency in dental plaque (85.7 to 94.4%). In contrast, all six of the periodontitis-related bacteria tested were detected in dental plaque (16.7 to 100%). These results indicate that the bacterial compositions in cardiovascular tissues were distinct from those in dental plaque, with only selected species found in the cardiovascular region, including *S. mutans*, *T. denticola*, and *A. actinomycetemcomitans*.

Identification of bacterial species. We also semiquantitatively analyzed the various bacterial components in the cardiovascular specimens using a broad-range PCR method with sequencing analysis. As for the heart valve tissues, streptococcal species were detected in 77.8% of the subjects and were found to account for 59.4% of the bacterial population (Table 3). Among them, *S. mutans* was found to be significantly prevalent, with a detection frequency of 77.8% in the subjects and was prevalent in 40.0% of them. Other streptococcal species, including *S. sanguinis*, *S. oralis*, *S. gordonii*, *S. pneumoniae*, *S. mitis*, *S. thermophilus*, *S. vestibularis*, *S. salivarius*, and *S. cristatus*, were also detected in 38.9% of the subjects and were prevalent in 19.4% of the populations, though each of those species had a significantly lower detection frequency than *S. mutans*. Other species were also identified, though their incidence rates were far below that of *S. mutans*. Dental plaque samples from the same subjects were also quantitatively analyzed, and various species also found in the heart valve tissues

TABLE 3. Identification and quantification of bacterial species harbored in heart valve tissues and dental plaque samples

Species[a]	Result (%) for specimen type[b]			
	Heart valve ($n = 35$)		Dental plaque ($n = 18$)	
	Quantity	Frequency	Quantity	Frequency
Streptococcus	59.4	77.8	21.7	77.8
S. mutans	40.0	77.8	2.8	16.7
Other streptococci	19.4	38.9	18.9	77.8
Nonstreptococci				
Burkholderia	9.1*	22.2*		
Stenotrophomonas	4.3*	8.5*		
Ralstonia	4.0*	20.0*		
Prevotella	1.4*	5.7*	6.1	33.3
Staphylococcus	1.1*	11.4*		
Capnocytophaga	1.1*	5.7*		
Corynebacterium	0.9*	2.9*		
Neisseria	0.6*	11.1*		
Capnocytophaga			12.2	72.2
Fusobacterium			10.0	55.6
Corynebacterium			9.4	50.0
Neisseria			6.1	33.3
Actinomyces			2.2	11.1
Porphyromonas			1.1	11.1
Tannerella			0.6	5.6
Others[c]	18.1*	54.2*	30.6	94.4

[a] Only species with quantities greater than 0.5% are listed.
[b] *, significant difference ($P < 0.05$) compared to *S. mutans*.
[c] Samples containing species unspecified in the databases.

TABLE 4. Identification and quantification of bacterial species harbored in atheromatous plaque and dental plaque samples

Species[a]	Result (%) for specimen type[b]			
	Atheromatous plaque (n = 27)		Dental plaque (n = 14)	
	Quantity	Frequency	Quantity	Frequency
Streptococcus	81.1	88.9	20.0	64.3
S. mutans	48.1	77.8	4.3	28.6
Other streptococci	33.0	59.3	15.7	57.1
Nonstreptococci				
Staphylococcus	3.7*	3.7*		
Stenotrophomonas	3.3*	7.4*		
Prevotella	3.3*	3.7*	10.0	57.1
Burkholderia	1.9*	11.1*		
Ralstonia	1.1*	3.7*		
Capnocytophaga			10.7	64.3
Neisseria			9.3	35.7
Fusobacterium			6.4	35.7
Corynebacterium			5.0	28.6
Porphyromonas			4.3	28.6
Actinomyces			2.9	28.6
Tannerella			2.1	14.3
Others[c]	5.6*	25.9*	29.3	85.7

[a] Only species with quantities greater than 0.5% are listed.
[b] *, significant difference ($P < 0.05$) compared to *S. mutans*.
[c] Samples containing species unspecified in the databases.

were detected. Although streptococcal species were frequently detected, their quantities were quite different from those in the heart valve specimens. In addition, periodontitis-related bacteria were detected in significant quantities and frequency in dental plaque, whereas their presence in heart valve tissue was shown to be negligible by this method.

We also semiquantitatively analyzed the atheromatous plaque specimens (Table 4). Similar to those from the heart valve, streptococcal species were most frequently detected (frequency, 88.9%; quantity, 81.1%), with *S. mutans* the most predominant species (77.8% and 48.1%, respectively). The incidence of other species, including periodontitis-related bacteria, was negligible compared to that of *S. mutans*. From the results of our analysis of dental plaque samples, the bacterial profiles for frequency and quantity were found to be significantly different from those of atheromatous plaque.

DISCUSSION

S. mutans is known to be one of the pathogens that causes IE, though it is primarily a major cariogenic pathogen that is a normal inhabitant of the oral cavity in most individuals. Unexpectedly, *S. mutans* was detected at high frequencies and quantities in both heart valve tissues and atheromatous plaque samples in the present study, as a simple PCR analysis detected it significantly more frequently than *T. denticola* (Tables 2), which is a periodontal bacterium most frequently detected in atherosclerotic lesions (17). In addition, our semiquantitative analysis revealed that *S. mutans* was significantly prevalent in both quantity and frequency in heart valve and atheromatous plaque specimens, as compared to the other tested species, and while the amounts of periodontitis-related bacteria including *T. denticola* were negligible (Tables 3 and 4). Although there are limits to interpreting our semiquantitative analysis results from 10 clones for each sample in this study, the broad-range PCR and sequencing method used is able to identify all of the species registered in available databases, which supports our finding that *S. mutans* was more frequently detected than any other species, in addition to the results of the simple PCR method. Therefore, it is possible to speculate that *S. mutans* is a causative agent for cardiovascular disease.

Periodontitis-related bacteria have been identified in atheromatous samples using PCR in several studies, in which the detection rates varied, likely due to differences in the DNA extraction methods employed. Haraszthy et al. (7) reported the detection frequencies of periodontitis-related bacteria in 50 atheromatous specimens, which included cytomegalovirus (38%), *Chlamydia pneumoniae* (18%), *T. forsythia* (30%), *P. gingivalis* (26%), *A. actinomycetemcomitans* (18%), and *P. intermedia* (14%). Another study that analyzed carotid endarterectomy samples by immunostaining showed that *C. pneumoniae*, cytomegalovirus, herpes simplex virus 1, *P. gingivalis*, and *S. sanguinis* were positive, with frequencies ranging from 9% to 64% (4). In addition, *T. denticola* was detected in 23% of DNA samples of formalin-fixed, paraffin-embedded atherosclerotic lesions, such as thoracic and abdominal aneurysms (17). However, oral streptococcal species were not targeted for detection in those studies, and to the best of our knowledge, this is the first study to analyze streptococcal profiles in both dental plaque and cardiovascular tissue specimens from the same subjects. *T. denticola* was the most frequently detected species among six major periodontitis-related bacteria (Table 2), though our broad-range PCR assays revealed that *S. mutans* was predominant compared to the other tested periodontitis-related bacteria in aneurysm tissues.

Systemic challenge by *P. gingivalis* was reported to accelerate atherogenic plaque formation in a murine model (11). One of the crucial steps for the development of atheromatous plaque lesions is formation of foam cells, which are macrophages that accumulate from excess cholesterol, and *P. gingivalis* has been shown to enhance their formation (10). Interestingly, that study also showed that *S. mutans* strain GS-5 possessed properties similar to those of *P. gingivalis*. In addition, *P. gingivalis* and *S. mutans* were shown to induce platelet aggregation, which presumably leads to thrombus formation (8), while it was also recently found that *S. mutans* cells bind to extracellular matrix molecules and fibrinogen with contribution from the major surface protein antigen PAc (3). It is unknown if *S. mutans* can invade endothelial cells and form atheromatous lesions, though those previous findings suggest an etiological involvement of the bacterium in cardiovascular diseases. Additional studies are necessary to provide the biological rationale for the involvement of this cariogenic pathogen.

The broad-range PCR and direct sequencing as well as conventional PCR techniques used in this study are reliable methods also utilized for clinical diagnosis of IE (18, 19). From the present findings obtained with those methods, an important question is the source of *S. mutans* found in cardiovascular tissues. This bacterium is a normal oral inhabitant that may migrate to cardiovascular endothelial tissues. In previous reports, several blood isolates of *S. mutans* from IE patients were shown to possess cariogenic properties and were considered to be oral derivatives (14, 15). In future studies, it will also be

necessary to determine if the *S. mutans* organisms found in cardiovascular tissues are cariogenic strains. In addition, though diagnoses for cardiovascular diseases are well characterized, there is no specific classification of disorders related to bacterial occurrence. Thus, extensive investigations are needed to answer these critical questions.

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